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(54) Title: COMPOUND AND METHOD FOR THE RETARDATION OF COLLAGEN CROSS-LINKING

(57) Abstract

The present invention provides a method for reducing or preventing collagen cross-linking in skin and/or damage to skin cell DNA. The method comprises treating the skin with a composition including an antioxidant compound. The antioxidant compound is selected from the group consisting of carnosine, homocarnosine, anserine, 3-methyl-L-histidine, L-alanyl-L-tyrosine, acyl homocarnosine, acetyl carnosine, iodo carnosine, di-iodo carnosine, anserine nitrate, carbenoxylone carnosine, analogues thereof and combinations thereof. The antioxidant compounds of choice are carnosine and homocarnasine with preference for carnosine.

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"COMPOUND AND METHOD FOR THE RETARDATION OF COLLAGEN CROSS-LINKING"

Field of the Invention

The present invention relates to a method for reducing or preventing collagen cross-linking in skin and/or damage to skin cell DNA. In particular the present invention relates to the use of specific dipeptides and analogues thereof which have the capability of decreasing or preventing collagen cross-linking either during aging and/or following exposure to UV radiation. The method of the present invention is also applicable for decreasing DNA damage due to UV radiation.

Background of the Invention

Oxidative stress and tissue damage by active oxygen species has been suggested as the basis for a number of diseases including cancer and aging (Halliwell and Gutteridge, 1985; Harman, 1987; Saul et al, 1987).

The levels and formation of highly reactive free radicals are known to be enhanced by ultraviolet radiation. Such reactive species can subsequently react with DNA, RNA, proteins and lipids. Natural defence mechanisms to such reactive and potentially damaging species are believed to exist and numerous molecules with anti-oxidant properties have been identified in tissues (eg vitamin E, carnosine and ascorbic acid). However, the physiological role of carnosine and its analogues in vivo remains unclear.

Other roles suggested for carnosine, in addition to being an efficient singlet-oxygen scavenger, include neutralization of lactic acid (Davey, 1960), a copper chelator (Brown, 1981), an activator of myosin ATPase (Parker and Ring 1980) and a regulator of enzymes (Ikeda et al 1980).

During the aging of mammalian skin the quantity of mature collagen cross-links increases, whereas the number

of immature, reducible cross-links decreases (Ames, 1983). Although the nature of cross-links in other tissues (eg. tendon) may differ, the same general trend exists (Ames, 1983; Rattan et al, 1982). The rates of change in the amounts of cross-links for various species appears to reflect the differences in life span for the different species. For instance, the amount of one mature cross-link HHL (histidinohydroxylysinonorleucine) rises in a linear fashion in human skin up to age 40 years whereas in bovine skin the amount of HHL plateaus at 4 years (Kohen et al, 1988). Cross-links arise from precursor lysine (Vizioli et al, 1983) or hydroxylysine (Boldyrev et al 1987) residues in collagen chains which are oxidatively deaminated. The aldehydes produced then condense with with similar residues to give aldols or with adjacent lysine or hydroxylysine residues to give Schiff-based compounds. The degree of cross-links in collagen also increases on UV irradiation. Thus there is a correlation between the rate of collagen cross-links and the rate of aging of skin and possibly other tissues.

Summary of the Invention

The present invention consists in a method for reducing or preventing collagen cross-linking in skin and/or damage to skin cell DNA comprising treating the skin with a composition comprising a suitable excipient in combination with an active compound, the active compound being selected from the group consisting of carnosine, homocarnosine, anserine, 3-methyl-L-histidine, L-alanyl-L-tyrosine, acyl homocarnosine, acetyl carnosine, iodo carnosine, di-iodo carnosine, anserine nitrate carbenoxylone carnosine, analogs thereof and mixtures of two or more of the foregoing.

In a preferred embodiment of the present the active compound is a naturally occurring (e.g. found in human tissue) anti-oxidant compound such as carnosine

vitamin C and Vitamin E.

Detailed Description of the Invention

In order that the nature of the present invention may be more clearly understood, preferred forms thereof will now be described with reference to the following examples.

Example 1

Mouse Skin Experiments

An example of the collagen cross-linking inhibitory activity of carnosine is shown in Table 2 and the effects of UV irradiation on total reducible species in mouse skin in Table 1.

L-carnosine is available from the Sigma Chemical Company or BDH Chemicals Ltd. Poole, England. ChromAr HPLC grade acetonitrile was obtained through Mallinkrodt Australia Pty. Ltd. and all solvents were filtered and degassed before us. $KB(H^3)_4$ was purchased from CEA France, through the Australian Atomic Energy Commission, with a specific activity of 50-70 Ci/mmol.

Dermal fibroblasts were cultured from primary explants of tissue derived from Swiss mice. The cells were maintained throughout in Dulbecco's Modified Eagles Medium (Gibco) and supplemented with 10% foetal bovine serum (Cytosystems Pty. Ltd.) and used between second and third passages. Skin sections were also obtained from neonatal Swiss mice. Sections were trimmed of as much subcutaneous material as possible and rinsed briefly in phosphate-buffered saline before following experimental procedure. All samples were incubated in varying concentrations of L-carnosine in phosphate-buffered saline (PBS) for 1 hour at 37°C prior to UV treatment. Prior to reduction with borohydride, samples were washed extensively in PBS at 40°C to remove as much as possible of contaminating protein, glycoprotein and glycosamino glycans with minimal disruption of the collagen structure.

Both skin sections and open dishes of dermal

(β-alanyl-L-histidine).

At present it is preferred that the active compound is carnosine or homocarnosine on a combination thereof and most preferably carnosine.

In a further preferred embodiment of the present invention the active compound is linked to another molecule, which molecule is such that the composition is improved in regard to skin permeation, skin application and tissue absorption. It is preferred that this other molecule is an amino acid or peptide.

The method of the present invention will generally involve topical application of the composition to the skin, however, the composition may be injected subcutaneously or intramuscularly or may be taken orally. The concentration of the active compound in the composition will depend upon the route of administration and may be at the direction of a physician, however, it is expected that the concentration of the active compound would be in the range of 1 to 100 mg per ml of a skin cream formulation for instance, the preferred range would be from 3 to 20 mg/ml.

It is believed the method of the present invention will reduce aging of the skin by decreasing or preventing collagen cross-linking due to exposure to UV radiation or sunlight. Given that the method is also capable of preventing DNA damage as a result of UV radiation, the method of the present invention has applicability in the prevention of skin cancer.

The composition according to the present invention may include, in addition to the active peptide molecules discussed above, a non-peptide compound which can inhibit or prevent cross-linking of collagen. Such compounds which may be advantageously included in the present composition include bilirubin, carotenoids, mannitol, reduced glutathione, selenium, uric acid, vitamin A,

fibroblasts were exposed for three hours at 920 uW/cm using a 24 watt germicidal ultraviolet lamp. Samples were kept moist through the UV treatment.

Reduction with $KB(H^3)_4$ was carried out at room temperature in the same PBS buffer for 1 hour in the ratio of 100:1 wet weight of sample/borohydride. The reaction was stopped by the addition of 4M acetic acid to lower pH to 3.00. Samples were then dialysed against distilled water at 4°C until free of soluble radioactivity. Samples were hydrolyzed in Sequanal grade 6N hydrochloric acid under nitrogen for 22 hours at 110°. Each sample was then rotary evaporated to dryness twice from distilled water before being taken up in distilled water. Hydrolysates were neutralized with 0.5M NaOH before HPLC.

HPLC was performed on a Pharmacia HPLC/FPLC system comprising a P3500 HPLC pump, LCC500 programmer, and using a heated column compartment.

Initial separations were performed on a Brownlees 25 cm x 0.46 cm Amino-Spheri 5 column at a temperature of 35°C and an eluent flow rate of 1.0 ml/minute. Column life was considerably lengthened by the use of a Brownlee 4.6 x 30 mm Amino-Spheri 5 pre-column guard cartridge, and a Brownlee 15 x 32 mm Anion Newguard in the solvent line. Brownlee columns were supplied through Activon Scientific Products Co Pty. Ltd. Australia.

The gradient system comprised two solvents. Solvent A contained 10 mM-potassium phosphate buffer, pH 4.3, and solvent B contained HPLC-grade acetonitrile diluted 50:7 (v/v) with water. Amino acids could be separated by using a gradient programme similar to that of the prior art, but the programme was modified for separation of reduced collagen components.

Programme 1 was as follows: 1.95% solvent B for 5 min; 2, linear gradient 95% - 70% solvent B over 15 min; 3, linear gradient 70% - 50% solvent B over 15 min; 4,

50% solvent B for 10 min; 5, linear gradient 50% - 95% solvent B over 5 min.

Fractions of volume 0.5 ml were collected directly into 20 ml scintillation vials and 5.0 ml of Amersham PCSII high-efficiency phase combining scintillant was added. Counting was done in an LKB 1215 Rack beta II instrument.

TOTAL REDUCIBLE SPECIES IN YOUNG MOUSE SKIN

TABLE 1

Tissue	U.V. Treatment	Total (3H) CPM
Skin section		
Skin (exterior)	-	10000
Skin (exteror)	+	24000
Skin (interior)	-	11000
Skin (interior)	+	27000
Skin cells		
Fibroblasts	-	19000
Fibroblasts	+	38000

2mg samples were treated with normal light or U.V. light for 2.5 hours then reduced with KB(3H)4 and hydrolyzed prior to HPLC.

TABLE 2

REVERSAL OF UV INDUCED COLLAGEN CROSS-LINKS BY CARNOSINE

Tissue	UV Treatment	Carnosine	Total (3H) CPM
Skin sections	-	-	15000
	+	-	23000
	+	+	14000
Skin cells	-	-	14000
	+	-	20000
	+	Low dose (2mM)	14000
		High dose (10mM)	10000

Samples were treated as in Table 1 with or without protection by carnosine solution.

Example 2

Dermal Fibroblasts Experiments

Primary cultures of mouse dermal fibroblasts (MDF) were isolated from new born mouse skin and were serially passaged in DMEM medium with high glucose plus 10% foetal calf serum for no more than four passages before experimentation. Prior to UV exposure, cell layers were washed with 50 mls of PBS to remove all traces of growth medium. The MDF were then incubated with 25 mls of PBS with or without 10 mM carnosine for 1 hour at 37°C. All the procedures were carried out with minimum exposure to outside light.

At the end of the incubation period the replicate cell populations were exposed to 0,2,4 or 6 minutes of UV A light. Following irradiation the cell monolayers were scraped and transferred to centrifuge tubes containing PBS. After centrifugation the supernatant was removed and the cell pellets resuspended in 30 mls of PBS. All samples were then washed for 3 days at 4°C. The PBS changed twice daily.

The samples were then reduced with $KB[^3H]_4$ and acid hydrolysed. The hydrosylates were rotary evaporated, neutralised and lyophilised before HPLC analysis (Smolenski et al, 1983 Biochem J., 213, 525-532). HPLC fractions of 0.5 mls were collected directly into 20ml scintilation vials and 5ml of Amersham PCS II high efficiency phase combining scintillant added. Counting was carried out in a LKB 1215 Rack Beta II. The results of these Experiments are shown in Figures 1-6.

Mouse dermal fibroblasts were scraped and washed in PBS. The isolated cell suspension was then processed for HPLC using the methods described.

The profile shown in Figure 1 is a representation of a typical non ultraviolet irradiated sample (CONTROL). The fractions in region 40 to 90 consist of isolated

reducible collagen crosslinks. The difference in peak height is indicative of the amount of a particular type of crosslinking amino acid complex present in the sample.

The profile shown in Figure 2 depicts the alteration in isolated crosslinks after a 2 minute exposure to UV A light. The major peak at fraction no. 55 has disappeared and there has been a positional shift of the peak at fraction. 83 to 87.

It is speculated that these differences represent modifications of the crosslinking amino acid complexes due to interaction of free radicals generated by the action of UV on water molecules.

As shown in Figure 3 after 4 minutes exposure to UV A light the profile of reducible crosslinks is showing a large increase in crosslinking amino acid complexes in the region from fraction 55 to 75.

As shown in Figure 4, after 6 minutes of UV A exposure there is a marked change in the content of reducible crosslinking amino acid complexes present in fractions 55 to 75. This probably results from modifications of the less complex crosslinks leading to an accumulation in fractions 55 to 75 and possibly the formation of entirely new crosslinks due to the interaction of free radicals.

Figure 5 shows the results obtained when mouse dermal fibroblasts are exposed for 6 minutes to UV A in the presence of 10 mM carnosine. As can be seen there is a marked difference in the profile of isolated crosslinks in comparison to that shown in Figure 4.

Figure 6 shows a comparison of profiles produced when MDF cells were exposed to 2 minutes UV A or to 6 minutes UV A but in the presence of 10 mM carnosine. It is obvious from this overlay of profiles that carnosine has protected the MDF cells from the effects of UV A as seen by the decrease in peaks from fractions 55-75.

Summary

When 10 mM carnosine is present in the bathing solution over MDF during exposure to UV A there is a 70% reduction in the formation of altered reducible crosslinks produced. This is exemplified by the appearance of the HPLC profile i.e. the 6 minute UV A exposure + 10 mM carnosine profile resembles that of the 2 minute UV A exposure profile.

Example 3

Human Fibroblasts (MRC-5) with UV A Light Experiments

The human lung fibroblast cell strain (MRC-5) was used to examine the degree of protection carnosine would give to human collagen when exposed to UV A light.

Fibroblasts are responsible for the maintenance of connective tissue in animals. They actively secrete collagen propeptides into the interstitial spaces, a proportion of which are deposited into the extracellular matrix as connective tissue collagen.

A similar protocol to that used for the MDF cells was employed in these experiments, however, the UV A exposure time was increased to 15 minutes. This was done to increase the collagen crosslinking changes that had been evident with the 6 minute UV A exposure of MDF cells. The results of these experiments are shown in Figures 7-10.

In Figure 7, populations of MRC-5 at similar cell numbers to that of MDF were scraped and processed for HPLC. These cells were not treated with UV A i.e. - CONTROL - the profile was similar to that produced by the MDF cells but there were differences. The major collagen crosslinking peaks were in the region from fractions 40-90.

Figure 8 shows the HPLC profile obtained when MRC-5 cells were exposed to 15 minutes UV A light in the presence of 10 mM carnosine. The profile is similar to that of the control (Fig. 7) and very different from the 15 minute UV A exposure in the absence of carnosine

(Fig. 9).

Figure 9 shows the profile produced when MRC-5 cells were exposed to 15 minures UV A light. The region from fractions 40 through 90 are extensiv ly increased indicating a putative incorporation of newly produced crosslinking complexes into the cellular collagen.

Figure 10 shows an overlay of HPLC profiles isolated from the MRC-5 control and from the MRC-5 after 15 minutes exposure to UV A in the presence of 10 mM carnosine. Again, there is a great deal of similarity between the two profiles indicating that carnosine is protecting the collagen from free radical attack.

Summary

The results from these experiments also supports the premise that carnosine protects collagen against UV A induced crosslinking. This is shown by the remarkable similarity between the MRC-5 control HPLC profile and that of the MRC-5 cells that have been exposed to 15 minutes UV A in the presence of carnosine. Also, the marked changes in the profile obtained from the MRC-5 cells that had UV A exposure but no carnosine present would indicate that carnosine is extremely effective in its protection of collagen against crosslinking. These experiments which had a 150% increase in UV A exposure time still demonstrated a greater than 70% protection with 10 mM carnosine.

Example 4

Rat Tail Tendon Experiments

Isometric melting has been widely used to determine a number of age related changes in collagen (Mitchell and Rigby, 1975 BBA, 393, 531-541). Robins and Bailey (1975 Biochem J., 149, 381-385) have proposed that the density of collagen crosslinks is constant with time but as ageing occurs, the labile reducible aldimine bonds formed from lysine and hydroxylysine, are converted to a thermally

stable, non-reducible bond which accounts for the age related collagen changes.

This method of isometric melting was used to examine how UV light may alter the collagen crosslinking. Rat tail tendon, which had been used for a number of other collagen ageing studies (Mitchell and Rigby, 1975 BBA, 393, 531-541; Rigby and Mitchell, 1978, BBA, 532, 65-70 and BBA, 544, 62-68; Rigby et al, 1977, BBRC, 79(2), 400-405 was used in these experiments.

This method has many advantages over other methods as it is a direct measure of age related crosslinking changes. As the present application has relevance to photoageing and in particular collagen crosslinking by UV light this method makes it possible to make quantitative measurements of the extent of UV crosslinking with time. Further, this method enables the effectiveness of carnosine and other antioxidants in protecting the tendons against UV induced free radicals to be determined.

Materials and Methods

The technique of isometric melting was applied to the rat tail tendon in order to determine the effects of photoageing.

Ninety day old Sprague-Dawley rats were used in these experiments.

Isolation of Tendons

The tail was removed by excision and transported to the laboratory in ice. the tendons were then carefully dissected out on a saline moistened surgical pad to prevent drying. Each tendon was then measured and cut in half. The top half of the tendon was used for experiments while the bottom was kept at $4^{o}C$ as a physical control.

UV Irradiation and Isometric Measurement

The experimental half of the tendon was incubated at $4^{o}C$ in the appropriate test solution for 20 hours prior to UV exposure. Following this exposure the tendons were

washed in PBS and kept at 4°C until analysed.

The isometric apparatus consisted of a strain gauge - Shinkho transducer type UL-100gm connected to an amplifier. After attachment to the strain gauge the sample was immersed in a jacketed pyrex bath containing PBS. During the experiments the bath was heated with a Tamson circulating water heater. To measure the temperature increase a FLUKE thermocouple model 80TK was fixed to a region next to the tendon attachment site. Measurements of force and temperature were recorded simultaneously with an ICI DP600 dual pen chart recorder.

For analysis the tendon was re-cut to a standard length of 3cm before attachment to the isometric apparatus. The attached tendon was then immersed into the bath of PBS at 20°C and a tension of 1 gram applied. After a relaxation period of 15 minutes the apparatus was turned on and the temperature increase at a rate of 1°C per minute until melting occurred. Final measurements were then obtained from the chart recorder.

To determine the exposure time needed to produce a measurable change in tendon crosslinking a time course experiment was carried out. The results of this experiment are shown in Figure 11. It was found that an exposure of 180 minutes using a light source that consisted of 4 UV A tubes and 2 UV B tubes gave a reproducible result. This time period was used for the following experiments.

Carnosine, homo carnosine and anserine were used to pretreat replicate tendons. These were then exposed to UV AB for 180 minutes The results from these experiments are shown in Figure 12. In these experiments carnosine and homocarnosine at 10 and 100 mM effectively protected the tendons against UV induced crosslinking. Whilst no protective effect was observed with anserine at 10 mM it is believed that anserine may provide a protective effect

at higher concentrations. (Unfortunately anserine waS nOt tested as higher concentrations due to its lack of availability).

Figure 13 shows the results obtained when a set of other di and tri peptides were examined for their ability to protect tendons against UV induced crosslinking in comparison to carnosine. It was found that none of those tested protected tendons against UV induced crosslinking. Two of the peptides contained histidine and still were inactive. These results suggest that carnosine is probably acting via its antioxidant property to protect against UV induced collagen crosslinking.

Figure 14 shows comparison of tendon data from Figure 12 with Sm measurements for glutathione at 10 mM (10GSH). Glutathione appears more effective using this method of measurement. Anserine (10A) is not working at this concentration. It is important to note that glutathione would not be available to act as free radical scavenger in vivo at this concentrations.

Summary

The results from these experiments using isometric melting techniques prove conclusively that carnosine acts effectively in protecting the rat tail tendon against UV induced collagen crosslinking. Also from the results of the other di and tri peptides tested, it is also obvious that the carnosine effect is specific and occuring because of its antioxidant property.

CLAIMS:

1. A method for reducing or preventing collagen crosslinking in skin and/or damage to skin cell DNA comprising treating the skin with a composition comprising a suitable excipient in combination with an active compound, the active compound being selected from the group consisting of carnosine, homocarnosine, anserine, 3-methyl-L-histidine, L-alanyl-L-tyrosine, acyl homocarnosine, acetyl carnosine, iodo carnosine, di-iodo carnosine, anserine nitrate, carbenoxylone carnosine, analogues thereof and combinations thereof.

2. A method as claimed in claim 1 in which the active compound is carnosine or homocarnosine or a combination thereof.

3. A method as claimed in claim 2 in which the active compound is carnosine.

4. A method as claimed in any one of claims 1-3 in which the active compound is linked to another molecule, which molecule is such that the composition is improved in regard to skin permeation, skin application and tissue absorption.

5. A method as claimed in claim 4 in which the molecule is an amino acid or peptide.

6. A method as claimed in any one of claims 1-5 in which the method involves topical application of the composition to the skin.

7. A method as claimed in any one of claims 1-6 in which the composition includes a compound selected from the group consisting of bilirubin, carotenoids, mannitol, reduced glutathione, selenium, uric acid, vitamin A, vitamin C, vitamin E and combinations therof.

8. A method for reducing or preventing collagen crosslinking in skin due to exposure to UV light, said method comprising treating the skin with a composition comprising a suitable excipient in combination with an

active compound, the active compound being selected from the group consisting of carnosine, homocarnosine, anserine, 3-methyl-L-histidine, L-alanyl-L-tyrosine, acyl homocarnosine, acetyl carnosine, iodo carnosine, di-iodo carnosine, anserine nitrate, carbenoxylone carnosine, analogues thereof and combinations thereof.

9. A method as claimed in claim 8 in which the active compound is carnosine or homocarnosine or a combination thereof.

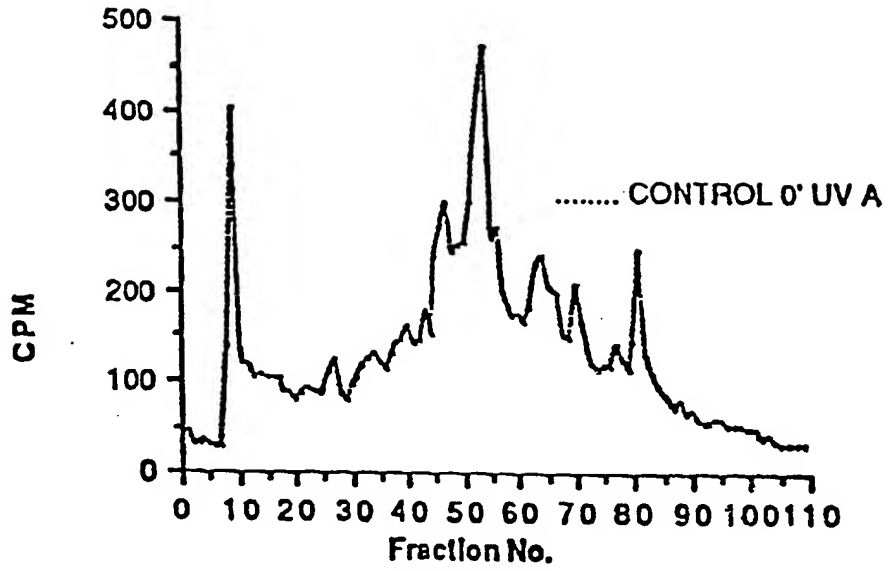
10. A method as claimed in claim 9 in which the active compound is carnosine.

11. A method as claimed in any one of claims 8-10 in which the active compound is linked to another molecule, which molecule is such that the composition is improved in regard to skin permeation, skin application and tissue absorption.

12. A method as claimed in claim 11 in which the molecule is an amino acid or peptide.

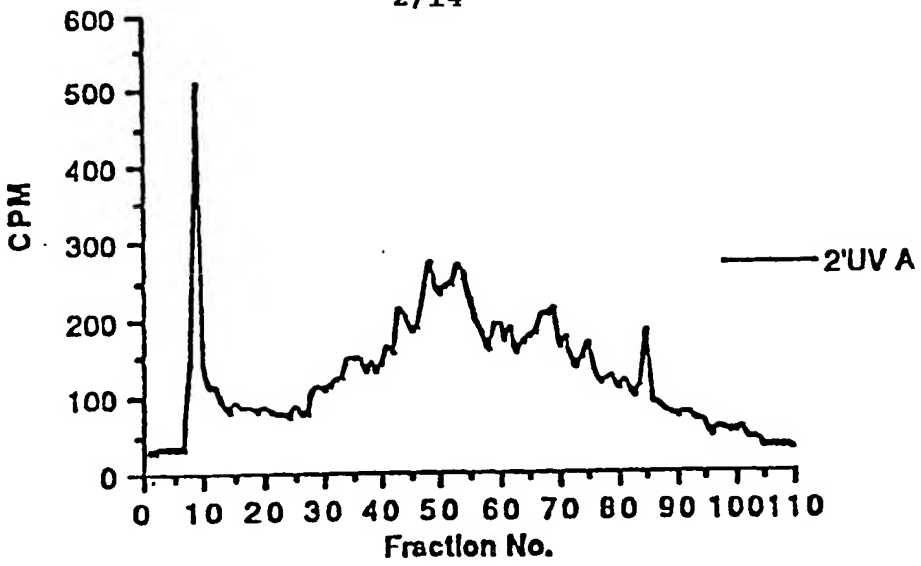
13. A method as claimed in any one of claims 8-12 in which the method involves topical application of the composition to the skin.

14. A method as claimed in any one of claims 8-13 in which the composition includes a compound selected from the group consisting of bilirubin, carotenoids, mannitol, reduced glutathione, selenium, uric acid, vitamin A, vitamin C, vitamin E and combinations therof.



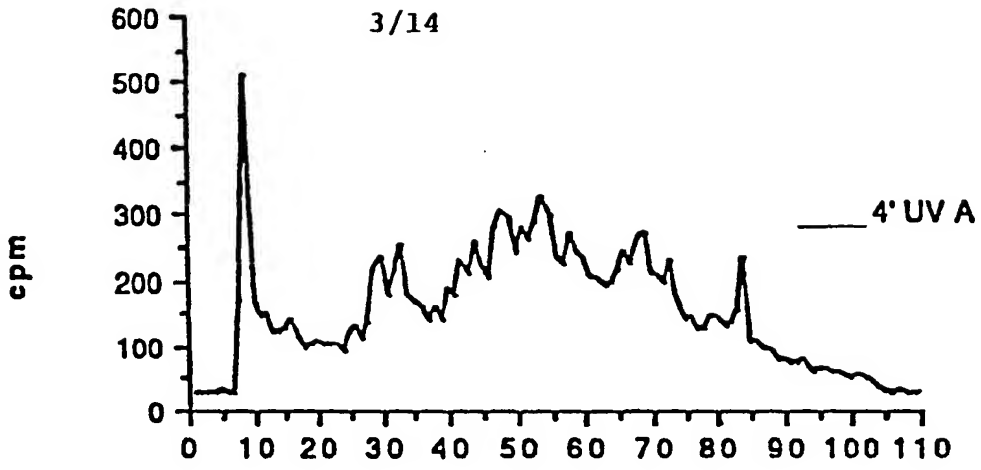
HPLC profile of mouse dermal fibroblasts after 0' exposure to UV A ie.CONTROL.

Figure1.



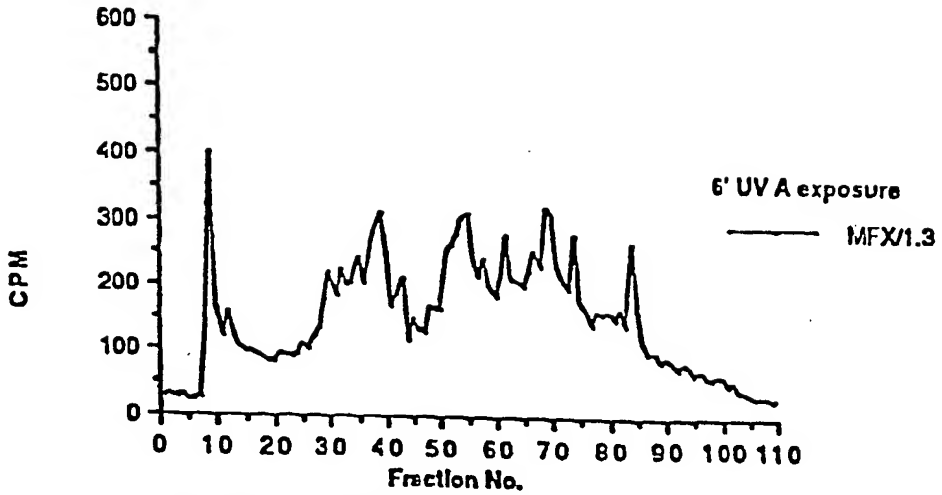
HPLC profile of mouse dermal fibroblasts after 2' exposure to UV A.

Figure,2.



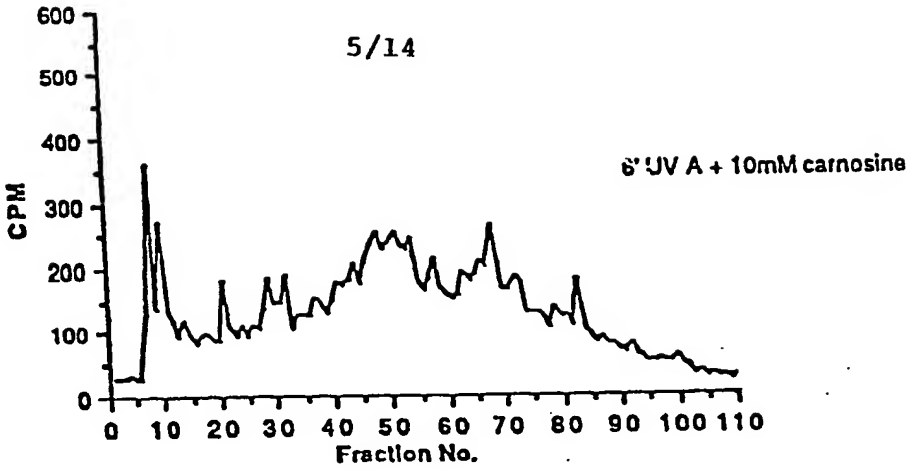
HPLC profile of mouse fibroblasts after a 4' UV A exposure.

Figure ,3.



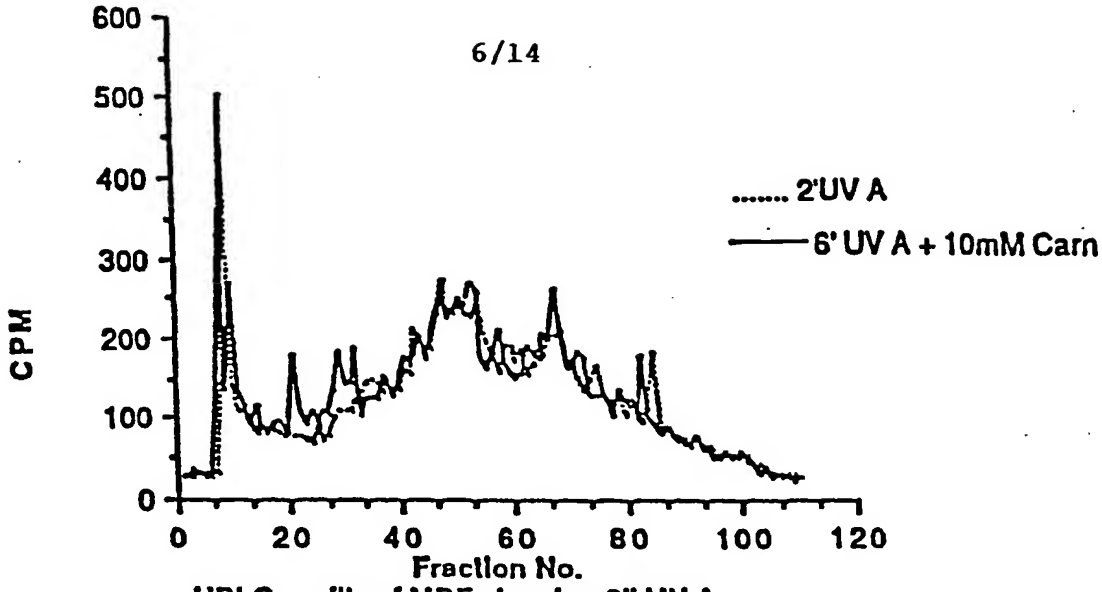
Mouse Dermal Fibroblasts after 6' exposure to UV A light (HPLC profile of crosslinks).

Figure,4.



HPLC profile of MDF after 6' exposure to UV A with 10mM carnosine added.

Figure,5.



HPLC profile of MDF showing 2" UV A exposure overlayed with 6' UV A exposure but with 10mM carnosine present.

Figure, 6.

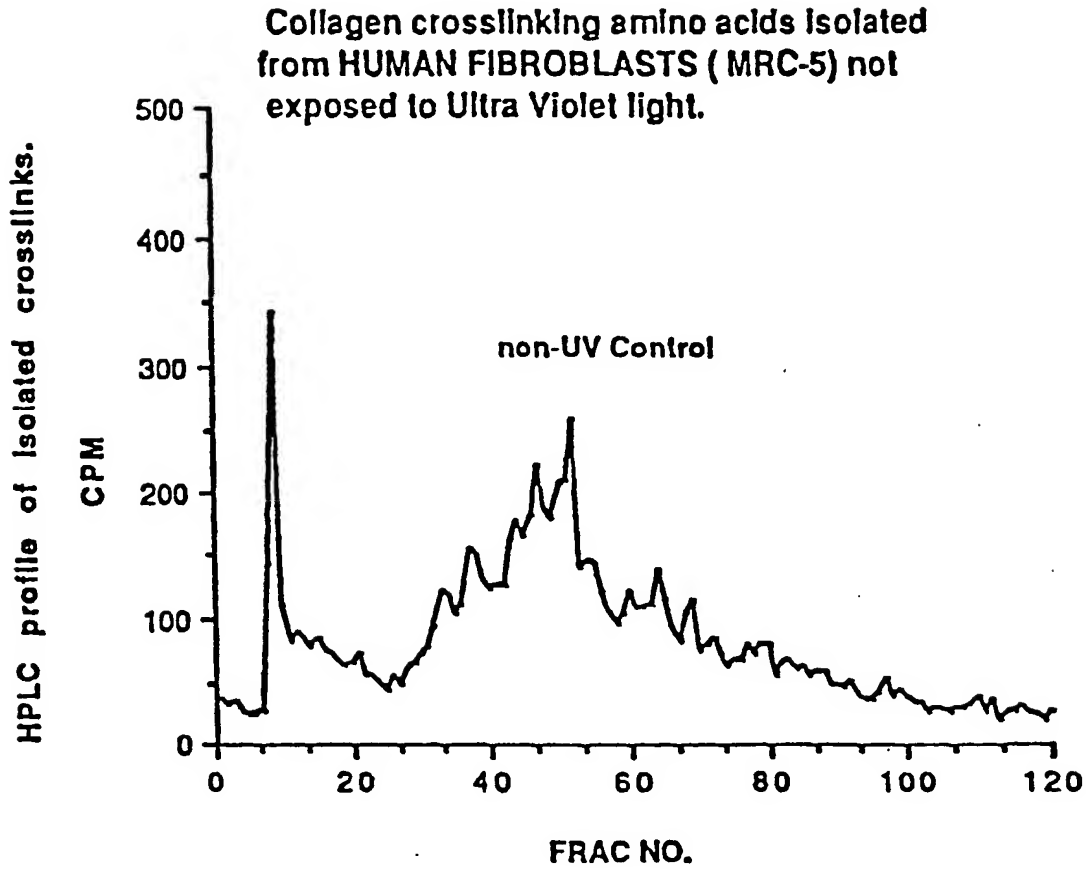
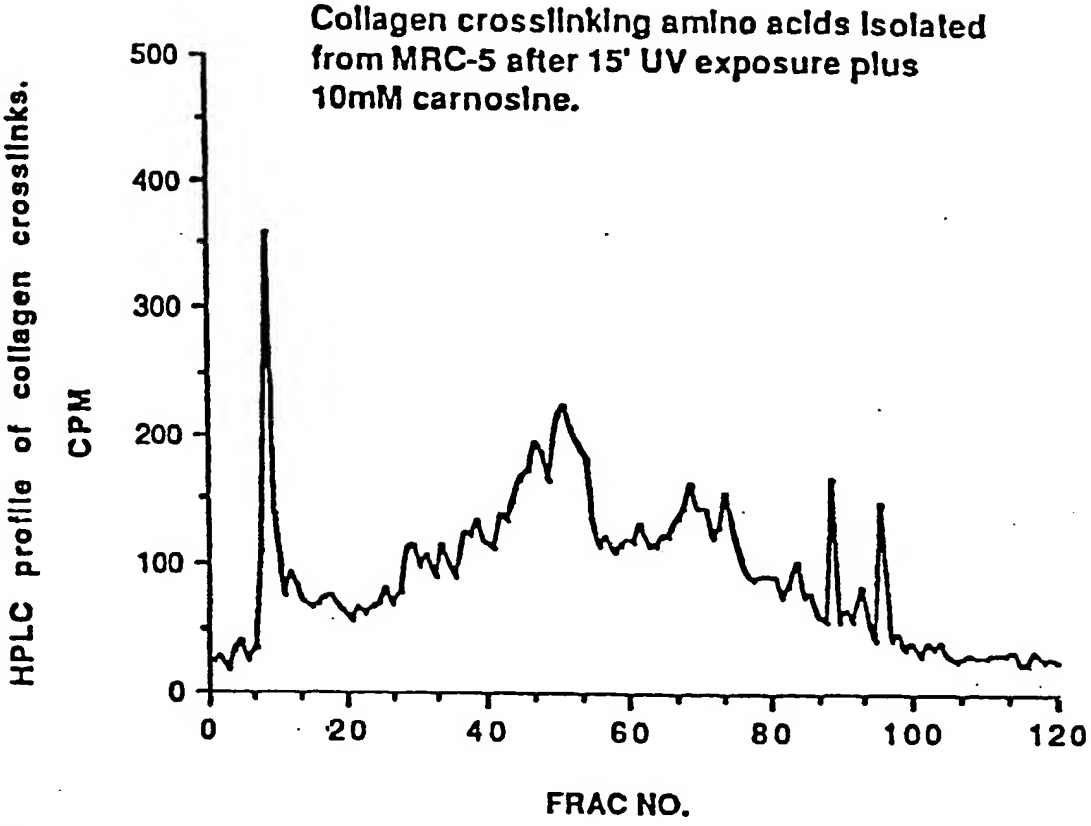

Collagen crosslinking amino acids isolated from HUMAN FIBROBLASTS (MRC-5) not exposed to Ultra Violet light.
HPLC profile of isolated crosslinks.
CPM
500
400
300
200
100
0
non-UV Control
0
20
40
60
80
100
120
FRAC NO.

Figure ,7.


Collagen crosslinking amino acids isolated from MRC-5 after 15' UV exposure plus 10mM carnosine.
HPLC profile of collagen crosslinks.
CPM
500
400
300
200
100
0
0
20
40
60
80
100
120
FRAC NO.

Figure,8.

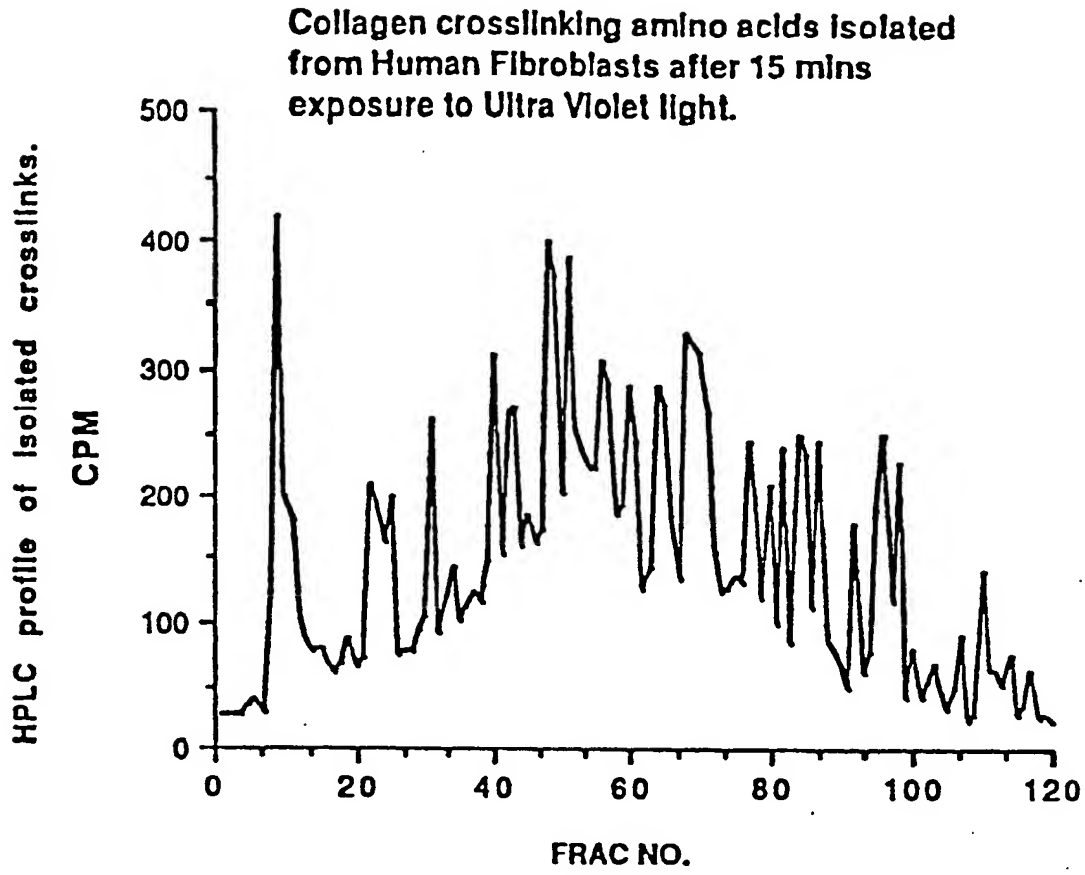
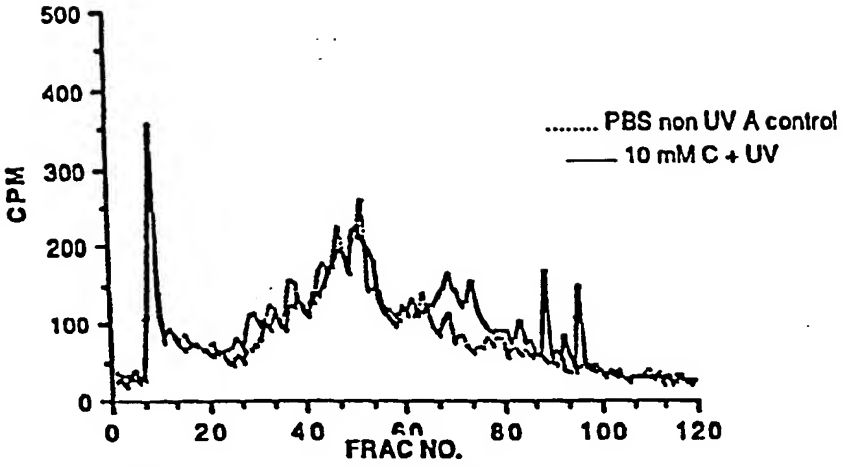
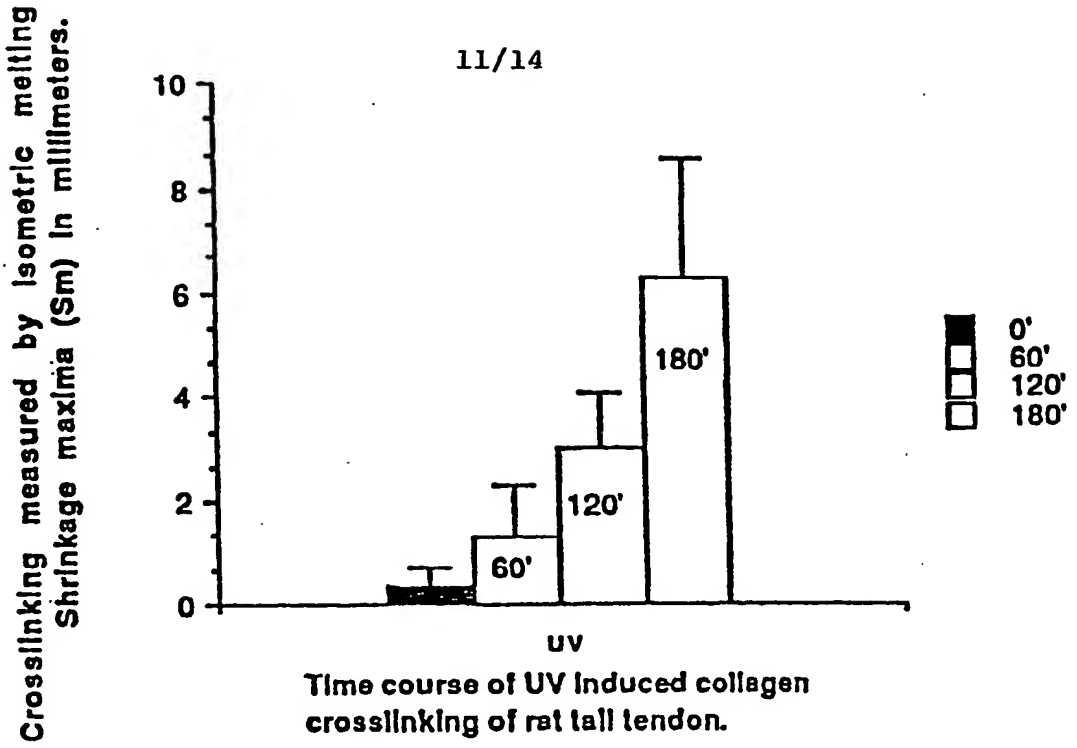


Figure.9.



HPLC profile of MRC-5 non UV A control overlayed with 15' UV A in the presence of 10mM carnosine.

Figure,10.



Time course of UV induced collagen crosslinking of rat tail tendon.

FIGURE,11.

The reduction of UV induced collagen crosslinking in Rat Tail Tendon by Carnosine and its analogues.

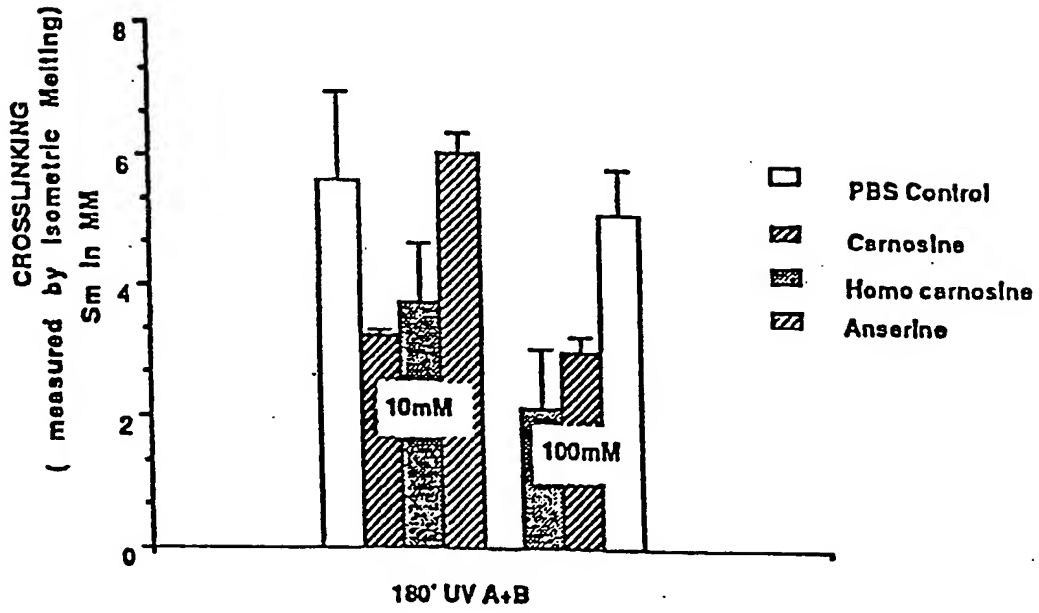


FIGURE 12

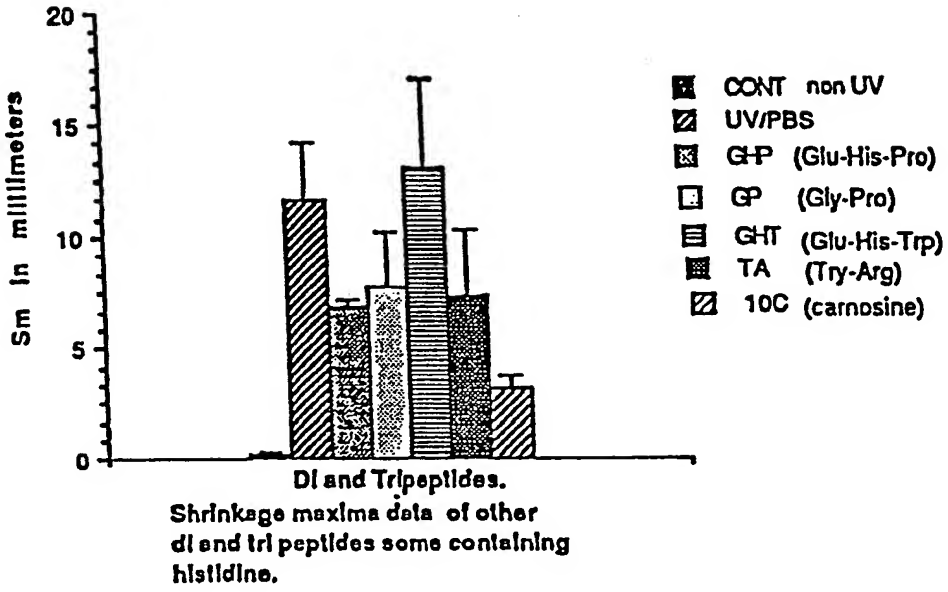

20
15
10
5
0
Sm in millimeters
CONT non UV
UV/PBS
GHP (Glu-His-Pro)
GP (Gly-Pro)
GHT (Glu-His-Trp)
TA (Try-Arg)
10C (carnosine)
DI and Tripeptides.
Shrinkage maxima data of other di and tri peptides some containing histidine.

Figure ,13.

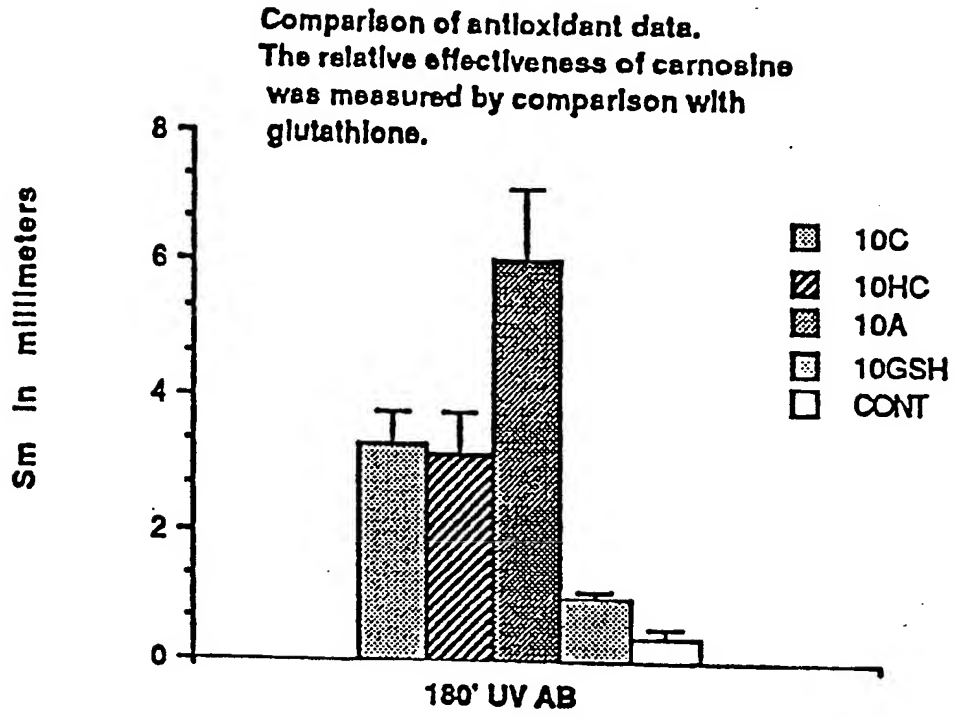


FIGURE 14

INTERNATIONAL SEARCH REPORT

International Application No. PCT/AU 89/00422

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] A61K 7/40, 7/48, 37/02

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC	A61K 7/40, 7/48, 37/02

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

AU: IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
A	Derwent Abstract Accession no. 83-810824/45, Class B04, JP,A, 58-164516 (NAGAI K.), 29 September 1983 (29.09.83)	
A	DE,A, 3424781 (NAGAI KINESHIROU), 17 January 1985 (17.01.85)	
A	GB,A, 2143732 (NAGAI KINESHIRO), 20 February 1985 (20.02.85)	

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
5 January 1990 (05.01.90)	16 January 1990
International Searching Authority	Signature of Authorized Officer
Australian Patent Office	STEVEN CHEW

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 89/00422

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members
GB 2143732	DE 3424997 JP 60016926

END OF ANNEX